Altered TP receptor function in isolated, perfused kidneys of nondiabetic and diabetic ApoE-deficient mice

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The number of patients suffering from end-stage renal disease has more than doubled over the past 10 years. To stop this progression, risk factors and early stages of chronic kidney disease need to be evaluated (7). Atherosclerosis is associated with renal dysfunction, but the presence of lesions represents an advanced stage of the atherosclerotic process. Renal functional abnormalities, preceding the onset of ischemic nephropathy, may exist at early stages of atherogenesis (2, 7). An early endothelial dysfunction is observed not only in atherosclerosis but also in hypertension, hypercholesterolemia, and diabetes (15, 33). Vasoconstrictors can be responsible for the altered vascular responsiveness, and activators of receptors for thromboxane A2 (TXA2; TP receptors) are frequently involved (5, 43).

Treatment of atherosclerotic patients with a single dose of the TP-receptor antagonist Triplion (S 18886) restores altered endothelium-dependent vasodilatation (3). Similarly, in atherosclerotic rabbits (18) and diabetic apolipoprotein E-deficient (ApoE-KO) mice (46), the endothelium-dependent relaxations to ACh were preserved by a treatment with an antagonist of TP-receptor. Furthermore, in this latter model, a marked improvement of renal oxidant stress and proteinuria was observed following treatment with Triplion (41). TP receptors are present in renal tissues such as glomeruli and arteries (1, 21), and evidence for a role of vasoconstrictor eicosanoids in renal disease has already existed for over a decade (27). The activation of TP receptors is seldom associated with TXA2 but involves other activators such as isoprostanes or HETEs (3, 6, 46).

However, in humans with diabetic nephropathy and in murine models of this pathology (12, 14), TXA2 itself has been implicated as a mediator of increased glomerular permeability to albumin (12), although other TP receptor agonists may contribute to the pathogenesis of diabetic kidney disease (41, 46).

Murine models of atherosclerosis (ApoE-KO and LDL receptor KO mice) and of diabetic atherosclerosis (diabetic ApoE-KO mice) have been developed to evaluate atherogenesis and its consequences on the vasculature. Antagonists of TP receptors decrease atherosclerotic plaque formation in ApoE-KO mice (6) and in diabetic ApoE-KO mice (46). This beneficial effect of TP receptor antagonists was mimicked by the deletion of the TP receptor gene in mixed ApoE-TP receptor KO mice (20).

The purpose of the present work was to study the contribution of TP receptors to the vasoconstrictor and vasodilator mechanisms of the isolated and perfused murine kidney and to determine whether early alterations in these mechanisms could be observed in hypercholesterolemic ApoE-KO mice and in the preatherosclerotic diabetic ApoE-KO mice.

METHODS

Isolated, Perfused Mouse Kidney

Male ApoE-KO and their wild-type counterparts (C57BL6) mice 7–15 wk old (body weight 25.1 ± 0.2 g; n = 113) were purchased from Charles River, kept on a regular diet, and maintained in a humidity- and temperature-controlled room. All studies reported were performed in accordance with European Community Guidelines for the use of laboratory animals. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
the use of experimental animals and was approved by the ethical committee on Animal Experiments of the Servier Research Institute.

The experimental procedure was adapted from that used for the isolated, perfused rat kidney (10) and afterward was also used in our laboratory (17, 34). Mice were anesthetized with pentobarbital sodium (50 mg/kg ip). The abdomen was opened by midline incision, and the left renal artery was cannulated via an incision made in the aorta. Then, the kidney was perfused at a constant flow by a peristaltic pump (Gilson Minipuls), with a warmed (37°C) and oxygenated (95% O2-5% CO2) Tyrode solution of the following composition (in mM): 137 NaCl, 2.7 KCl, 1.8 CaCl2, 1.1 MgCl2, 12.0 NaHCO3, 0.42 NaHPO4, 0.026 Ca-EDTA, and 5.6 glucose. The perfused kidney was removed from the animal and placed in a perfusion chamber. The perfusate was not recirculated. The flow rate was adjusted to 0.9 ml/min (optimum flow rate), corresponding to 6 ml·min⁻¹·g⁻¹ kidney wt. The changes in renal vascular resistance were recorded as changes in perfusion pressure recorded downstream from the pump via a pressure transducer (P10 EZ, Statham) connected to data-acquisition system IOX (EMKA Technologies). Once the perfusion pressure reached its steady state, after a 60-min equilibration period, experiments were started. To add pharmacological agents to the perfusate, two different sites were used: the substance was added via the 8-iso PGF2α (10 pmol)-methoxamine (3 nmol)-8-iso PGF2α, (10 nmol)-ANG II (3 pmol)-ET-1 (3 pmol); 2) NE (0.3 nmol)-U 46619 (0.1 pmol)-PGH2 (1 nmol)-5-HT (1 nmol)-ET-1 (3 pmol); 3) AA (0.3 nmol)-NE (0.3 nmol)-U 46619 (0.1 nmol; this series was also tested in the presence of the cyclooxygenase inhibitor indomethacin (10 μM)).

To verify the specificity of the ET-1-mediated constrictions, experiments were performed in the presence of the selective ETA and ETB antagonists BQ-123 (1 μM) and BQ-788 (1 μM), respectively.

To verify the specificity of the response to AA, experiments were performed in the presence of the TXA2 synthase inhibitor dazoxiben (10 μM). In this series of experiments, the generation of TXA2 assay was quantified.

Part 3. Under the present experimental conditions, papaverine (10 nmol) did not affect basal perfusion pressure, indicating that the perfused murine kidney lacks intrinsic tone. Thus, to study vasodilator responses, a continuous renal vasoconstriction was produced by infusion of methoxamine (3 μM). During this response, vasodilator substances were injected as a bolus into the circuit with at least 10-min intervals.

Part 4. To study the mechanisms involved in the mouse renal vasodilator responses, studies using inhibitors or antagonists were performed. Bolus injection of ACh, nitric oxide (NO), PGI2, and atrial natriuretic peptide (ANP) were performed during a methoxamine-induced vasoconstriction in the presence of Triplion (10 nmol), indomethacin (10 μM), or BQ-123 and BQ-788 (1 μM), and the doses of the agonists used were similar to those described in part 3. Treatment with the antagonists or inhibitors started 30 min before the methoxamine infusion.

TXB2 assay. A bolus administration of AA (0.3 nmol) was performed, and 1-min fractions of renal effluent were collected over 5 min. The samples were stored at −20°C, and TXB2 was measured with a specific enzymatic immunoassay kit (Cayman Chemical, Spi Bio, France) according the instructions of the manufacturer. The levels of TXB2 are expressed in nanograms per milliliter.

Pathological Animals

Two mouse models of cardiovascular disease were studied: the ApoE-KO mouse and the diabetic ApoE-KO mouse.

Diabetes induction. Male ApoE-KO mice received a daily dose of streptozotocin (Sigma-Aldrich) in 0.05 M Na citrate, pH 4.5 (70 mg/kg ip), for 5 days at 7 wk of age, as described previously (46). Diabetes was confirmed by measurement of nonfasting plasma glucose levels (tail stick) before the experiment. To be categorized as diabetic, the mice had to have nonfasting plasma glucose levels >300 mg/dl. Three experimental groups of animals were then studied: C57BL6 nondiabetic control mice, ApoE-KO nondiabetic mice, and diabetic ApoE-KO mice. All animals were kept on a normal-chow diet ad libitum, and diabetic mice were maintained without additional insulin injections. Eight weeks later, the mice were used for the kidney perfusion experiments.

Biochemical measurements. At the time of death, blood samples were collected from the heart in Li-heparin tubes. The plasma concentration of total and HDL cholesterol and triglycerides were determined using a colorimetric kit (ABX). 8-ISO-PGF2α plasma levels were assayed by ELISA (Cayman Chemical). Vasoconstrictor agents. To compare renal vascular constriction among the control mice, the ApoE-KO mice, and the diabetic ApoE-KO mice, dose-response curves were performed in the isolated perfused kidney of the three groups. Two series of experiences were performed: I) NE (0.1 pmol–3 nmol), U 46619 (1 pmol–0.3 nmol), and 5-HT (10 pmol–3 nmol); and 2) 8-iso PGF2α (0.1–10 nmol), ANG II (0.1–30 pmol), and ET-1 (0.03–10 pmol).

To determine the involvement of TP receptors, some experiments were performed in the presence of Triplion (10 nM) or its vehicle. The following series of injection was used: 3) NE (0.3 nmol), U 46619 (0.1 nmol), and 8-iso PGF2α (0.1–10 nmol).
Vasodilator agents. To study vasodilator responses, a sustained renal vasoconstriction was produced by infusion of methoxamine (3 μM). During the vasoconstrictor responses, the following vasodilator substances were injected: ACh (3 pmol–0.3 nmol), NO (1–100 pmol), PGI₂ (10 nmol), and ANP (10 pmol).

Expression of TP receptors in kidneys by quantitative real-time PCR. The right kidneys of ApoE-KO and diabetic ApoE-KO mice were harvested under pentobarbital anesthesia. Total RNA was extracted using an RNeasy minikit (Qiagen). Frozen kidneys were disrupted and homogenized in 300 μl of RLT lysis buffer using a Mixer Mill MM 300 as described in the RNeasy minikit handbook (Qiagen). RT was performed with 1 μg RNA with a Superscript III first-strand cDNA synthesis kit (Invitrogen). Expression of TP receptors was quantified by real-time RT-PCR using the iCycler iQ Detection System (Bio-Rad). Each reaction was performed using the IQ SYBR Green supermix (Bio-Rad), 2 μl of cDNA, and 150 nM of each specific primer. Samples were denatured for 5 min and 30 s at 95°C and amplified for 40 cycles as follows: denaturation for 20 s at 95°C and annealing for 1 min at 63, 54, 54, and 56°C for the TP receptor, β-actin, hypoxanthine guanine phosphoribosyl transferase (HPRT), and GAPDH, respectively. Each real-time PCR run included cDNAs in duplicate in parallel with serial dilutions of cDNA mix tested for each primer pair to generate a linear standard curve (mean cycle threshold, defined as the cycle at which fluorescence was considered significantly greater than the background and within the linear range, plotted against the logarithm of the template concentration). This curve was used to estimate the relative quantity of the relevant mRNA in each sample. The values obtained with our samples were normalized to the geometric mean of the values obtained with three internal controls: HPRT, β-actin, and GAPDH. The generation of specific PCR products was confirmed by melting curve analysis.

Primers used to amplify the TP receptor were 5′-GTC TTC GGG CTC ATA TTC-3′ (forward) and 5′- CTG AAC CAT CTT CTC CAC-3′ (reverse); for β-actin were 5′- AAG ACC TCT ATG CCA ACA CAG-3′ (forward) and 5′-AGC CAC CGA TCC ACA CAG-3′ (reverse); for GAPDH were 5′-GCC TTC CGT CCT ACC CAC C-3′ (forward) and 5′-TGC CGT CCT ACC CAC C C-3′ (reverse); and for HPRT were 5′-AGC TAC TGT AAT GAT CAG TCA ACC-3′ (forward) and 5′-AGA GGT CCT TTT CAC CAG-3′ (reverse). All of these primers were designed by Beacon Designer Software (Primer Biosoft).

Drugs

ACh chloride, ANG II, rat ANP, AA sodium, 5-hydroxytryptamine creatinine sulfate, indomethacin, methoxamine hydrochloride, NE bitartrate, and papaverine hydrochloride were obtained from Sigma-Aldrich; BQ-123 sodium and BQ-788 sodium were purchased from Calbiochem; human ET-1 was obtained from Neo-system; and dazoxiben and (3-(6R)-6-(4-chlorophenyl)sulfonyl) amino-2-methyl-5,6,7,8 tetrahydro-1-naphthalenyl) propanoic acid, sodium salt (S 18886; Triplion) were synthesized by G. Lavielle at the Institut de Recherches Servier. All drugs were dissolved in water except AA (Na2CO3, 0.1 M), dazoxiben (0.02 N NaOH), indomethacin, and BQ-788 (DMSO); 12(S)-HETE, 15(S)-HETE, 20-HETE, 8-iso-PGF2α, 8-iso-PGE2, 8-iso-PGF2α, PGH2, PGH2 and U 46619 (9,11-dideoxy-9α, 11α-methanoepoxy-PGF2α) were purchased from Cayman Chemical and dissolved in ethanol, except PGF2 in Na2CO3 (2 mM); further dilutions were performed in water.

The solution of NO was prepared as previously described (17). Briefly, glass bottles were filled with distilled water and were deoxynogenated with N2. After preparation of a saturated solution of NO, adequate amounts of this solution were added with a syringe to the glass bottles, which were then sealed. The stock solution was prepared intertemporarily. The final molar concentrations of the NO solutions were calculated on the basis that a saturated solution contains 4.6% NO.

Analysis of Results

Perfusion pressure was measured in millimeters Hg, and changes in renal vascular pressure were expressed as mmHg vs. basal perfusion pressure. Data are expressed as means ± SE. The maximal response (Emax) and the pD2 values, which correspond to the negative logarithm of the dose of agonist which evoked 50% of the maximal response, were obtained from a nonlinear sigmoid regression analysis for each dose-response curve (GraphPad Prism, GraphPad Software); n is the number of animals from which the kidneys were taken.

Differences among the groups were determined by one- or two-way ANOVA followed by a Bonferroni test in the case of significant ANOVA. To evaluate significant differences between means before and after dazoxiben or Triplion, a Student’s t-test for paired or unpaired observations was performed. P < 0.05 was considered as statistically significant.

RESULTS

Responses in Kidneys of Wild-Type Mice

Physiological parameters. The flow rate of 0.9 ml/min produced a constant pressure of 44.2 ± 1.5 mmHg (n = 113), corresponding to the basal perfusion pressure. The initial bolus injection of NE (0.3 nmol) caused an increase in perfusion pressure that averaged 95.0 ± 5.2 mmHg (n = 113).

Vasoconstrictor responses. Bolus injections of increasing doses of various vasoconstricting agonists were performed: α-adrenergic agonists, NE (10 pmol–3 nmol), methoxamine (10 pmol–30 nmol), 5-HT (10 pmol–1 nmol), ANG II (0.1 pmol–0.1 nmol), and ET-1 (0.03–10 pmol), as well as substances that activate TP receptors: U 46619 (1–30 pmol), AA (10 pmol–0.3 nmol), PGH2 (10 pmol–3 nmol), and the two isoprostanes 8-iso-PGF2α (0.1–10 nmol) and 8-iso-PGE2 (10 pmol–3 nmol). The activation of TP receptors was a potent and powerful vasoconstricting stimulus since U 46619 was only slightly less potent than ANG II and had a similar Emax than the α-adrenergic agonists. 8-ISO-PGE2 and PGH2 caused maximal constrictions equivalent to those induced by U 46619 (1–30 pmol), while those to AA and 8-ISO-PGF2α were smaller. Additionally, three different HETE compounds [12(S)-HETE, 15(S)-HETE, and 20-HETE] did not evoke any constrictor responses in the range of doses tested (n = 3, data not shown). The order of potency of the various substances tested toward the TP receptor was U 46619 > AA > PGH2 > 8-ISO-PGE2 > 8-ISO-PGF2α > 12(S)-HETE, 15(S)-HETE, and 20-HETE (Table 1).

At the concentration of 1 nM, the specific TP receptor antagonist Triplion partially inhibited the constrictions evoked by U 46619 (1–30 pmol) whereas at 10 nM the antagonist totally inhibited these responses (Fig. 1A). This latter concentration of Triplion inhibited the constrictions to single doses of U 46619 (0.1 nmol), 8-ISO-PGF2α (10 nmol), and PGH2 (1 nmol) without altering those caused by NE (0.3 nmol), methoxamine (3 nmol), 5-HT (1 nmol), ANG II (3 pmol), and ET-1 (3 pmol) (Fig. 1B). The combination of the antagonists of ETα and ETβ endothelin receptors BQ-123 and BQ-788 (1 μM), which virtually abolished the ET-1-induced constriction, did not affect that to U 46619 (data not shown).

The vasoconstriction caused by AA was blocked by Triplion and by the cyclooxygenase inhibitor indomethacin (10 μM), while the latter did not affect the constrictions evoked by the U 46619 (Fig. 2A). Additionally, the vasoconstriction evoked by AA (0.3 nmol), but not that by U 46619 or by NE, was partially
but significantly inhibited by the TXA₂ synthase inhibitor dazoxiben (10 μM; Fig. 2B). The bolus injection of AA (0.3 nmol) was associated with an increase in the release of TXB₂, the TXA₂ metabolite, which peaked between 2 and 3 min after AA administration. The basal and stimulated TXB₂ production was significantly inhibited by dazoxiben (10 μM) (Fig. 2C).

**Dilator responses during tonic vasoconstrictions induced by methoxamine.** All the vasodilators tested did not modify perfusion pressure when added under basal conditions (i.e., without the presence of vasoconstrictors). In the presence of the α₁-adrenergic agonist methoxamine (3 μM), ACh (3 pmol–0.3 nmol) evoked biphasic responses; the first was a dose-dependent dilatation followed by a second phase of dose-dependent constriction (Fig. 3A), while PGI₂ (10 nmol) first caused a vasoconstriction that was followed immediately by a vasodilatation (Fig. 4A). Authentic NO (1–100 pmol),

![Fig. 1. Effect of the thromboxane A₂ (TP) receptor antagonist Triplion on vasoconstrictor responses in isolated, perfused mouse kidneys.](image)

![Fig. 2. Effect of the cyclooxygenase inhibitor indomethacin and the TXA₂ synthase inhibitor dazoxiben on vasoconstrictor responses (A and B) and on the production of TXB₂ (C) in isolated, perfused kidneys of C57BL/6 mice.](image)

**Table 1. Maximal responses and pD₂ values of vasoconstrictor substances in isolated, perfused mouse kidneys**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Eₘₐₓ, mmHg</th>
<th>pD₂</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>200±13</td>
<td>9.63±0.13</td>
<td>9</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>238±26</td>
<td>8.48±0.12</td>
<td>9</td>
</tr>
<tr>
<td>5-HT</td>
<td>91±16</td>
<td>9.38±0.36</td>
<td>6</td>
</tr>
<tr>
<td>ANG II</td>
<td>134±9</td>
<td>11.3±0.1</td>
<td>6</td>
</tr>
<tr>
<td>ET-1</td>
<td>264±51</td>
<td>11.5±0.2</td>
<td>6</td>
</tr>
<tr>
<td>U 46619</td>
<td>212±14</td>
<td>10.4±0.1</td>
<td>10</td>
</tr>
<tr>
<td>PGH₂</td>
<td>234±30</td>
<td>9.32±0.14</td>
<td>9</td>
</tr>
<tr>
<td>AA</td>
<td>150±25</td>
<td>9.88±0.22</td>
<td>7</td>
</tr>
<tr>
<td>8-iso-PGE₂</td>
<td>219±18</td>
<td>9.02±0.10</td>
<td>5</td>
</tr>
<tr>
<td>8-iso-PGF₁×₂</td>
<td>101±19*</td>
<td>ND</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are shown as means ± SE; n equals the number of animals used. 

Eₘₐₓ, maximal responses; 5-HT, 5-hydroxytryptamine; AA, arachidonic acid; ND, not determined. The pD₂ value for 8-iso-PGF₁×₂ was not calculated because maximal constriction was not obtained. *Increase in perfusion pressure at the highest dose of 8-iso-PGF₁×₂ used (10 nmol).

AA administration. The basal and stimulated TXB₂ production was significantly inhibited by dazoxiben (10 μM) (Fig. 2C).
Table 2. Maximal decreases in perfusion pressure caused by vasodilator substances during constrictions with methoxamine with or without inhibitors: influence of Triplion and indomethacin

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Triplion (10 nM)</th>
<th>Indomethacin (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 pmol</td>
<td>-3.6±2.6 (10)</td>
<td>-8.3±4.6* (6)</td>
<td>-0±0 (6)</td>
</tr>
<tr>
<td>10 pmol</td>
<td>-14.3±3.0 (10)</td>
<td>-26.2±6.2* (6)</td>
<td>-9.0±3.4 (6)</td>
</tr>
<tr>
<td>100 pmol</td>
<td>-36.0±2.7 (10)</td>
<td>-55.5±5.1* (6)</td>
<td>-35.0±6.1 (6)</td>
</tr>
<tr>
<td>ANP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 pmol</td>
<td>-43.9±4.5 (12)</td>
<td>-48.2±3.6 (6)</td>
<td>-40.7±5.1 (6)</td>
</tr>
<tr>
<td>Papaverine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nmol</td>
<td>-37.9±2.6 (20)</td>
<td>-57.5±4.6* (6)</td>
<td>-36.0±3.1 (6)</td>
</tr>
</tbody>
</table>

Values are shown as means ± SE expressed in mmHG, with the number of animals in parentheses. NO, nitric oxide; ANP, atrial natriuretic peptide. *P < 0.05 vs. control (ANOVA).

vs. control), but they were decreased by indomethacin (10 μM; 68 ± 5 mmHg, n = 6, P < 0.05 vs. control).

The dilator responses to ACh were not altered by indomethacin but were potentiated by Triplion (Fig. 3). Both drugs inhibited the ACh-induced vasconstrictions (Fig. 3). Combined blockade of ETA and ETB receptors by BQ-123 and BQ-788 did not alter the ACh-induced responses (data not shown).

The vasodilatations caused by PGI2 were not modified by indomethacin or Triplion (Fig. 4). However, the PGI2-induced vasconstrictions were enhanced by indomethacin and abolished by Triplion (Fig. 4). BQ-123+BQ-788 did not alter the PGI2-induced constriction (data not shown).

The vasodilator response to NO and papaverine were not modified by indomethacin (Table 2) or by BQ-123+BQ-788 (data not shown), but they were potentiated by Triplion (Table 2), whereas those to ANP were not altered by any of the treatments used (Table 2).
Pathological Models

The body weight, kidney weight, triglycerides, as well as total and HDL cholesterol of ApoE-KO mice were significantly higher than those of C57BL6 mice. Eight weeks after the induction of diabetes, blood glucose levels were markedly increased in diabetic mice. Their body weight was significantly lower than those of the two other strains, whereas the ratio of kidney weight to body weight was significantly higher. Total and HDL cholesterol were further augmented in diabetic ApoE-KO mice, but the ratio of HDL to total cholesterol showed a large decrease in HDL cholesterol in nondiabetic (26%) or diabetic ApoE-KO (16%) compared with C57BL6 mice (69%). Finally, the plasma levels of 8-iso-PGF$_{2\alpha}$ were significantly higher in diabetic ApoE-KO mice than in the two other groups (Table 3).

Vasoconstrictor responses in nondiabetic or diabetic ApoE KO mouse kidneys. The amplitude of the renal response to the vasoconstrictors NE (0.1 pmol–3 nmol), 5-HT (10 pmol–3 nmol), U 46619 (1 pmol–0.3 nmol), 8-iso-PGF$_{2\alpha}$ (0.1–10 nmol), ANG II (0.1 pmol–0.3 nmol), and ET-1 (0.03–10 pmol) was not affected in the kidneys of ApoE-KO compared with C57BL6 mice except for a small but significant decrease in the constrictor response induced by NE (Fig. 5). Compared with ApoE-KO mice, an increase in the responses to NE at 0.3 and 1 nmol was observed in diabetic ApoE-KO mice; the constric- tions were restored to the level of control mice. The constric- tions to 5-HT were decreased for diabetic compared with nondiabetic ApoE-KO mice; the responses to ET-1 and ANG II were not altered (Fig. 5 and Table 4).

The concentration-response curve for isoprostane 8-iso-

Table 3. Physiological and pathophysiological parameters: body and kidney weight, kidney/body weight ratio, and levels of glucose, triglyceride, total cholesterol, HDL cholesterol, and 8-iso-PGF$_{2\alpha}$ in blood or plasma of control, nondiabetic, and diabetic ApoE-KO mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Nondiabetic ApoE</th>
<th>Diabetic ApoE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.6±0.3 (36)</td>
<td>30.2±0.3* (33)</td>
<td>21.5±0.6†† (37)</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.16±0.003 (36)</td>
<td>0.20±0.005* (33)</td>
<td>0.19±0.005* (37)</td>
</tr>
<tr>
<td>Kidney/body weight (10$^{-3}$)</td>
<td>6.0±0.11 (36)</td>
<td>6.6±0.16 (33)</td>
<td>8.9±0.26*†† (37)</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>124±4.3 (36)</td>
<td>130±4 (33)</td>
<td>470±13*† (37)</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>65.3±5.3 (36)</td>
<td>108±10* (33)</td>
<td>119±16* (37)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>71.0±4.2 (36)</td>
<td>410±43* (33)</td>
<td>784±63* (37)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>48.7±3.0 (36)</td>
<td>107±23* (33)</td>
<td>124±8* (37)</td>
</tr>
<tr>
<td>8-iso-PGF$_{2\alpha}$, pg/ml</td>
<td>63.4±5.9 (10)</td>
<td>52.4±3.6 (9)</td>
<td>114±19* (8)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with the number of animals in parentheses. ApoE, apolipoprotein E; ApoE-KO, ApoE deficient. *P < 0.05 vs. control mice. †P < 0.05 vs. nondiabetic ApoE-KO mice.

Fig. 5. Dose-dependent vasoconstrictions induced by bolus injections of NE (n = 7, A), 5-HT (n = 7, B), ANG II (n = 5, C), and ET-1 (n = 5, D) in kidneys from nondiabetic ApoE deficient (ApoE-KO), diabetic-ApoE-KO (●), and age-matched control mice (■). Values are means ± SE. *P < 0.05 vs. control mice. #P < 0.05 vs. diabetic ApoE-KO mice.
specific TP receptor antagonist Triplion totally inhibited the constrictions evoked by U 46619 (0.1 nmol) and by 8-iso-PGF$_{2\alpha}$ (0.1–10 nmol) without altering those to NE (0.3 nmol; 144.4 ± 6.8 and 144.9 ± 7.4 mmHg, n = 6, P = NS) (Fig. 6).

**Vasodilator responses in nondiabetic or diabetic ApoE-KO mouse kidneys.** Methoxamine (3 μM) induced similar tonic vasoconstrictions in the three different groups of kidneys (72.8 ± 3.2 mmHg, n = 7, 63.9 ± 4.0 mmHg, n = 6, and 82.8 ± 6.6 mmHg, n = 8, for C57BL6 and nondiabetic or diabetic ApoE KO mice, respectively).

ACh (3 pmol–0.3 nmol) caused biphasic responses in the three groups of mice. The dilator responses were comparable for the three different groups; however, the dose-dependent constrictor responses evoked by ACh were significantly lower in diabetic ApoE-KO than in nondiabetic ApoE-KO or C57BL6 mice (Table 4).

The biphasic responses to PGI$_2$ (10 nmol), the dose-dependent vasodilatations to authentic NO (1–100 pmol), as well as the vasodilations to either ANP (10 pmol) or papaverine (10 nmol) were similar in the three different groups of mice (Table 5).

**DISCUSSION**

The present study shows that, in murine kidneys, the activation of TP receptors is a potent vasoconstrictor stimulus, U 46619 exhibiting pD$_2$ and $E_{\text{max}}$ values close to those of ANG.

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**Table 4. pD$_2$ values of vasoconstrictor substances in control, nondiabetic, and diabetic ApoE-KO mice**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Control</th>
<th>Nondiabetic ApoE</th>
<th>Diabetic ApoE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>9.46±0.07 (7)</td>
<td>9.30±0.06 (7)</td>
<td>9.55±0.13 (8)</td>
</tr>
<tr>
<td>U 46619</td>
<td>10.64±0.12 (7)</td>
<td>10.48±0.14 (8)</td>
<td>10.90±0.15 (8)</td>
</tr>
<tr>
<td>5-HT</td>
<td>9.31±0.31 (7)</td>
<td>9.18±0.28 (7)</td>
<td>9.18±0.39 (9)</td>
</tr>
<tr>
<td>8-iso-PGF$_{2\alpha}$</td>
<td>NC (9)</td>
<td>NC (8)</td>
<td>8.48±0.20 (9)</td>
</tr>
<tr>
<td>ET-1</td>
<td>11.80±0.14 (5)</td>
<td>11.83±0.16 (5)</td>
<td>12.08±0.12 (5)</td>
</tr>
<tr>
<td>ANG II</td>
<td>11.86±0.12 (5)</td>
<td>11.78±0.14 (5)</td>
<td>11.93±0.16 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with the number of animals in parentheses. The pD$_2$ value for 8-iso-PGF$_{2\alpha}$ was not calculated because maximal constriction was not obtained. NC, not calculated.
Table 5. Maximal variations in perfusion pressure induced by vasodilator substances during vasoconstriction caused by methoxamine in control, nondiabetic, and diabetic ApoE-KO mice

<table>
<thead>
<tr>
<th>Substance</th>
<th>Control (7)</th>
<th>Nondiabetic ApoE (6)</th>
<th>Diabetic ApoE (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO 1 pmol</td>
<td>−7.7 ± 5.0</td>
<td>−15 ± 6.7</td>
<td>−4.2 ± 1.6</td>
</tr>
<tr>
<td>NO 10 pmol</td>
<td>−16.1 ± 4.6</td>
<td>−20.8 ± 4.5</td>
<td>−13.4 ± 3.7</td>
</tr>
<tr>
<td>NO 100 pmol</td>
<td>−46.7 ± 3.4</td>
<td>−40.6 ± 3.9</td>
<td>−38.4 ± 3.8</td>
</tr>
<tr>
<td>ANP 10 pmol</td>
<td>−60.7 ± 9.2</td>
<td>−54.6 ± 4.0</td>
<td>−55.2 ± 4.6</td>
</tr>
<tr>
<td>PGH2 10 nmol</td>
<td>−44.8 ± 4.7</td>
<td>−33.3 ± 4.6</td>
<td>−42.6 ± 6.0</td>
</tr>
<tr>
<td>Papaverine 10 nmol</td>
<td>−51.6 ± 5.4</td>
<td>−46.5 ± 3.9</td>
<td>−44.2 ± 5.1</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as mmHg, with the number of animals in parentheses.

II, and that the activation of this receptor impedes the response of various vasodilators such as ACh, PGH2, and NO. Furthermore, in diabetic ApoE-KO mice, an enhanced vasoconstriction to TP receptor activation and especially in response to the isoprostane 8-iso-PGF2α, is observed.

The present work confirms that the isolated, perfused mouse kidney is a convenient model for the studying vasoconstrictor and vasodilator responses (38, 39). As reported for rat kidneys (10, 24, 35), the mouse renal vasculature is very sensitive to ET-1 and ANG II, and the reactivity to NE is comparable to that described for rat kidneys (10, 24). However, the mouse renal circulation responds less to 5-HT but more potently to AA than do rat kidneys (10, 26).

The first objective of our study was to examine the possible involvement of TP receptors in the renovascular reactivity to various vasoconstrictor agents. In the murine kidney, the potent and selective TP receptor antagonist Triplion (6, 31) blocked the responses evoked by U 46619, isoprostanes, and PGH2, indicating the activation of TP receptors. However, in contrast to rat kidneys, where the generation of unidentified TP receptor ligands contributes to NE-, ET-1-, and ANG II-induced constrictions (24, 30), in mouse kidneys these responses do not involve TP receptors. In the present study, Triplion and indomethacin blocked AA-induced constrictions while dazoxiben markedly inhibited both AA-induced constrictions and the generation of TXA2, indicating that this latter prostaglandin is the major contributor of the effects of AA. In rat kidneys, exogenous AA causes also a cyclooxygenase- and TP receptor-dependent vasoconstriction, which is attributed to the formation of PGH2 since inhibitors of TXA2 synthase fail to inhibit it (26, 29).

In mouse kidneys, ACh evokes biphasic responses, dilatations followed by constrictions. In preliminary experiments, we have shown that inhibitors of NO synthase, Nω-nitro-l-arginine, and of soluble guanylate cyclase, ODQ, blocked ACh-induced dilatations, suggesting the implication of the NO/cGMP pathway, as also shown in rat kidneys (4, 17). ACh-induced vasoconstriction does not involve endothelin since the combined ETA and ETB receptor blockade did not affect it. ACh-induced constrictions share similar characteristics with endothelium-dependent contractions, as observed in arteries of ApoE-KO mice (45) or spontaneously hypertensive rat aorta (43). Being blocked by a cyclooxygenase inhibitor and TP receptor antagonist, they involve the generation of a prostanoïd, possibly again TXA2 (45). The dilatations to ACh are enhanced following TP receptor blockade with Triplion, which would be consistent with the hypothesis that the effect of an endothelium-derived contracting factor (EDCF) impairs the NO-dependent relaxation. However, the vasodilations to exogenously added authentic NO are also potentiated by the presence of Triplion. This raises the possibility of a tonic influence of TP receptors on renal vasoreactivity. Indeed, a basal dazoxiben-sensitive production of TXA2 is measurable in the effluent of the isolated, perfused murine kidney. Arguments against this hypothesis are that this TP receptor antagonist did not affect the contractile response evoked by methoxamine and did not significantly influence the relaxation evoked by ANP. In patients with coronary artery disease, the ACh-induced increase in forearm blood flow also includes a component that involves the activation of TP receptors (3).

In murine kidneys, as reported in the rat (17), PGF2α caused also a biphasic response, although, in contrast to ACh, the constriction is observed first and is followed by a sustained dilatation. Again, the involvement of endogenous ET-1 does not contribute to the vasoconstriction since the ETA/ETB antagonists did not block it. Indomethacin did not inhibit the contractile response but paradoxically they enhanced the constriction, while Triplion blocked it. This indicates that the release of cyclooxygenase derivatives with overall vasorelaxing properties reduces the prostacyclin-induced contraction.

The constriction could be attributed to a direct effect of prostacyclin on the TP receptors. Prostaglandin and some of its analogs are preferential agonists of the IP receptor but can directly activate TP receptors and produce arterial contractions (19, 36). The second relaxation phase evoked by prostacyclin should be attributed to the stimulation of IP receptors.

Therefore, this study shows that the murine isolated and perfused kidney is a reliable model that shares some characteristics with the rat perfused kidney. In murine kidneys, the activation of TP receptors is a potent vasoconstrictor stimulus that reduces the response of various vasodilators such as ACh, PGF2α, and NO.

Mouse models of atherosclerosis and diabetes may help in understanding renal dysfunction related to early disease stages. In these models, the protective effects of TP receptor antagonism on development of atherosclerosis were recently demonstrated (6, 41, 46). The hypercholesterolemic ApoE-KO mouse presents early atherosclerotic lesions comparable to human lesions (44), and they can be accelerated by hyperglycemia (41, 46). Nondiabetic ApoE-KO mice do not have normal glomeruli, but they often have lipid deposits and a mild degree of glomerulosclerosis. The ApoE-KO mouse is a specific atherosclerosis-prone mouse model with increased remnant lipoproteins. It is expected that these mice show some vascular abnormalities, further accelerated in the context of diabetes. In our models, the relatively short duration of hypercholesterolemia and diabetes produces only minor but visible alterations in the kidney tubular morphology without evidence of atherosclerosis (data not shown). Mesangial expansion is a key feature in the pathogenesis of numerous renal diseases, including diabetic nephropathy, involving the glomerulus. In ApoE-null mice, a lipid-independent effect on mesangial expansion has been described (8). When exposed to hyperglycemia, mesangial cells respond by cellular proliferation and

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matrix synthesis, and the early changes induced by the deficiency in ApoE are exacerbated. These morphological changes in the kidney (glomerulosclerosis, tubulointerstitial injury), even in the absence of any visible atherosclerotic lesions, can influence the vasoconstrictor/vasodilator responses.

In the present study, normal vasodilatations were noted in kidneys of ApoE-KO animals, regardless of whether the disease was aggravated by diabetes. Thus at early stages of the pathologies, vasodilator activity of renal resistance arteries appears preserved. Conflicting data exist for ApoE-KO mice where alterations of vasodilator activity or no changes have been observed (37, 42). Furthermore, an apparent normal overall endothelial vasodilatation might hide early and profound compensatory changes in the contribution of the various endothelial mechanisms involved (NO, prostacyclin, EDHF, EDCF) (13, 22). For instance, in the aorta of ApoE-KO mice, the endothelium-dependent (and NO-dependent) relaxations are altered only in segments with plaque lesions (11). In the mesenteric artery of ApoE-KO mice, the response to ACh is not altered (13) while in that of the streptozotocin-treated ApoE-KO mice an overall decreased response is attributed to a decreased EDHF component, and an increased EDCF component with a paradoxical increased compensatory NO component (13, 22). In the carotid artery of ApoE-KO mice fed with a Western-type diet, the ACh-induced relaxations are apparently unaltered, but a decrease in the NO component is compensated by the endothelial production of vasodilator prostaglandins (16). The transient vasoconstrictions induced by ACh are decreased in the diabetic animals while those to exogenously added prostacyclin are maintained. This suggests that the generation by ACh of a putative EDCF is diminished. To dissect the various mechanisms underlying the vasodilator response of the murine kidneys was beyond the scope of the present study. This matter must be specifically addressed in further work.

Nevertheless, without obvious changes in vasodilator responses, changes in responses to vasoconstrictors were observed in ApoE-KO and diabetic ApoE-KO mice. In ApoE-KO mice, constrictions to NE are decreased, which was also observed in arteries of hypercholesterolemic rabbits, early during atherosclerosis (33). An intriguing observation is that NE constrictions are increased in diabetic animals, suggesting that diabetes, but not atherosclerosis, causes augmented constrictor activity to NE. Nevertheless, the most profound change is associated with TP receptor activation in the diabetic animals. In diabetic mice, the responses to the TP-receptor agonist U 46619 were slightly augmented and those to the isoprostane mals. In diabetic mice, the responses to the TP-receptor agoniststrictor activity to NE. Nevertheless, the most profound change is mediated by the increase in lipids or by the diabetic milieu per se. By using a nonchemical method to produce a mouse model of insulin-deficient diabetes, Renard et al. (28) showed that this model is associated with larger vascular lesions in mice on chow diets and suggested that diabetes increases lesion initiation and is associated with development of advanced lesions. Elevated glucose in diabetes has been recognized to increase levels of AA metabolites, likely as a result of the increased vascular inflammation. In aging ApoE-KO mice, isoprostane levels are increased, suggesting an age-dependent increase in lipid peroxidation products (25). In each case, both diabetes and/or hyperlipidemia are responsible for the increased oxidative stress, and their association is responsible for the alterations that produce the dramatic acceleration of renal disease in diabetic ApoE-KO mice (41).

In conclusion, in ApoE-KO and diabetic ApoE-KO mice, normal vasodilator responses, but altered constrictor responses are observed, and the latter involve TP receptor activation. This suggests that in the very early stages of atherosclerotic and/or diabetic atherosclerotic disease, an enhanced TP receptor activation is an early marker of renal dysfunction, and this pathway is a possible contributor in the exacerbation of the disease.

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REFERENCES


